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Endophytic bacteria in Miscanthus seed: implications for germination, vertical inheritance of endophytes, plant evolution and breeding

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Abstract

With growing interest in the role of microbiomes, and symbionts in particular, the aim of this study was to determine the diversity of the bacterial endophyte population within Miscanthus and to ascertain the extent of vertical transmission via the seed. A great diversity of endophytic bacteria was found in all parts of the mature plant (rhizome, root, stem and leaf), and in seedlings grown from sterilized seed grown in sterile conditions. A total of three phyla and five families of bacteria were identified as cultures compared to 19 phyla and 85 families using 16S rDNA amplification and sequencing. Not all cultured bacteria could be identified by 16S rDNA, implying that the true diversity is even greater. More bacterial diversity was identified in sterile-grown seedlings than in all parts of the mature plant combined, 17 and 13 phyla, respectively, with 11 in common. Five phyla were present in all plant samples examined. Vertical transmission via the seed may therefore be a major source of endophytes in Miscanthus, presumably supplemented by ingress of soil bacteria as the plant grows. Bacteria identified from the mature plant were predominantly similar to known bacterial sequences in GenBank, but a small number from the stem and many from the seed were novel, potentially adapted to an *in planta* life cycle. Endophytic bacteria were found to form spores and other dense structures, and this provides a mechanism for long-term survival and seed transmission. The staining of germinating seeds identified bacteria at the root tip of the emerging radicle. We propose that seed transmission of bacterial endophytes requires adaptation of both plant and microbes, plays a role in germination and has evolutionary significance and implications for future plant breeding approaches, in Miscanthus and more widely.

Keywords: 16S rRNA gene, bacterial endophyte, bioenergy, C4 grass, endospore, genomic adaptation, microscopy, PhyloChip, sustainable agriculture, vertical transmission

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Introduction

Endophytic bacteria live within plant tissues, gaining nutrients and/or shelter without causing visible detriment to the host, and have been reported to confer a range of benefits to host plants (reviewed recently in Farrar *et al.*, 2014). Bacterial endophytes are ubiquitous among plant species and reported benefits to the host include increased growth rate, higher biomass, additional defences against invading pathogens, biological nitrogen fixation, phosphate solubilization, indole acetic acid production and reduction of contaminant-induced stress (James, 2000; Lee *et al.*, 2004; Rosenblueth & Martínez-Romero, 2006; Weyens *et al.*, 2009; Compant *et al.*, 2010). There is increasing interest in these symbiotic relationships (defined as originally described by de Bary in 1879 as 'the living together of unlike organisms'), both in terms of understanding their evolution, and exploiting components of them for plant production (Farrar *et al.*, 2014). Endophytes are generally believed to originate from outside the plant, a subset of epiphytes from soil populations or leaf surfaces, predominantly from the rhizosphere (Rosenblueth & Martínez-Romero, 2006). They have previously been demonstrated to enter the plant through spaces between root cells or junctions between root hairs and cells (James *et al.*, 2002; Hardoim *et al.*, 2008; Compant *et al.*, 2010), and this is presumed to be the predominant source of bacteria within the plant. However, bacteria have also been reported in the seed of various plant species (reviewed recently in Truyens *et al.*, 2015), which could represent an alternative route for plant colonization, with evolutionary consequence.

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Miscanthus, an undomesticated C4 grass native to South-East Asia, exhibits multiple traits important for a sustainable bioenergy crop. These include perenniality, rapid growth, high annual biomass yields with low fertilizer inputs and the ability to grow on low-quality land (Lewandowski *et al*., 2000, 2003; Heaton *et al*., 2008). Current research is aimed to improve biomass yield and ensure the crop is sustainable in terms of carbon and nutrient balance, and resilient to survive and produce high yields on marginal land over 10–15 years under variable and changing climatic conditions. Bacterial species offer one sustainable route to increasing yield and resilience in Miscanthus, and diazotrophic species including *Acetobacter diazotrophicus* (Gillis *et al*., 1989) *Herbaspirillum seropedicae* (Baldani *et al*., 1986) and Pantoea (Loiret *et al*., 2004) have been isolated from within surface-sterilized tissues of sugarcane, a close relative of *Miscanthus*. Fluorescently labelled strains have been imaged within sugarcane (Rouws *et al*., 2010) and other plants (Monteiro *et al*., 2012) following inoculation, and endophytic species have been demonstrated to fix nitrogen and boost plant growth in sugarcane and other plants (Elbeltagy *et al*., 2001; Muthukumarasamy *et al*., 2006; Oliveira *et al*., 2006; Loiret *et al*., 2009; Quecine *et al*., 2012; Wei *et al*., 2014). A number of endophytic bacteria have been isolated previously from within Miscanthus tissues: *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* (Olivares *et al*., 1996), *Azospirillum*-like, *Azospirillum lipoferum*-like, *Herbaspirillum*-like (Kirchhof *et al*., 1997), *Azospirillum doereinerae* sp. nov. GSF71 (Eckert *et al*., 2001), *Herbaspirillum frisingense* sp. (Kirchhof *et al*., 2001; Straub *et al*., 2013a) and *Clostridium* spp. (Miyamoto *et al*., 2004).

Several approaches are available to characterize a bacterial population. Originally, only readily culturable bacteria could be identified, but these are now considered to represent a subset of the total species richness present for most systems. Culture-independent methodology focuses on the amplification by PCR of the 16S rRNA gene which can be used to identify the species present and the diversity within a population (Amman *et al*., 1995). As with any PCR-based method, the results are only as good as the specificity of the primers to the target DNA sequence, which is difficult to estimate in a mixed sample of unknown species. An advance on this approach is to use a hybridization-based method such as a DNA chip to determine sequence homology at the 16S locus (DeAngelis *et al*., 2008). In all cases, identification is dependent on the current state of knowledge in the 16S RNA databases. As previously unstudied systems are characterized, the number of previously unidentified bacteria increases. In contrast with previous reports for Miscanthus which focused on the identification and characterization of a limited number of specific species or genera of bacterial endophytes, this study aimed to capture a more complete diversity of the endophytic bacteria species present within Miscanthus. To achieve this bacterial identification and characterization, four methods were adopted as follows: 1. 16S rRNA gene (rDNA) amplification of total DNA preparations from plant tissues followed by DNA sequencing, 2. PhyloChip analysis of 16S rDNA, 3. plate culture of bacterial colonies and 4. microscopy.

The aim of this study was to determine the presence and diversity of endophytic bacteria in Miscanthus leaves, stems, rhizomes, roots and seed (achieved using seedlings derived from surface-sterilized seed), to determine the extent of seed transmission of bacterial endophytes in Miscanthus, and establish a plausible mechanism by which this might be achieved.

Materials and methods

Sampling of plant tissue material

Seed and mature plant tissue were obtained from the *Miscanthus* germplasm collection at IBERS, Aberystwyth. Plant material used in this study was part of a diverse collection of 244 plants maintained and grown at IBERS (described previously by Jensen *et al*., 2011) grown in pots and in the field since collection. Seeds and mature tissues were sterilized by submerging in 40 ml of 10% bleach solution for 20 min with gentle agitation. The samples were then rinsed in 40 ml of distilled water before being submerged in 40 ml of 70% ethanol for 20 min and rinsed with sterile distilled water a final time. The final rinse was retained to test for residual bacteria by both culturing and PCR.

All mature tissues (leaves, stems, rhizomes and roots) were removed from mature pot grown *M. sinensis* plants of a single genotype using secateurs (sterilized with 70% ethanol between each cut). Whole leaf blades were removed at the ligule. Stems were cut from the internodal section of the stem between the uppermost 2 nodes for uniform sampling from the same area of stem regardless of stem length. Rhizomes were split from the base of the plant. Lateral roots were cut into lengths of 10 cm measuring from the root tip. After harvesting, plant tissue samples were cut into 1 cm lengths rinsed in sterile distilled water and surface-sterilized.

Chloroplasts were isolated from *M. sinensis* leaf tissue using the Sigma Chloroplast Isolation Kit (Sigma-Aldrich, Poole, UK). A total of 30 g (FW) of leaves was homogenized in 48 ml of an ice-cold isolation buffer using a blender, and the homogenate was filtered through mesh (supplied in kit: cat: F 6801, Filter Mesh 100) into 50 ml tubes. The filtrate was centrifuged for 7 min at 1000 *g* to sediment chloroplasts as a green pellet, which was then resuspended in isolation buffer. The chloroplast suspension was then loaded on top of a stepwise Percoll® gradient (40%/80%) for 15 min at 3200 *g*.

Sterilized seeds from an *M. sinensis* and an *M. sacchariflorus* genotype were evenly placed on 8 cm Petri dishes, eight per plate. The plates contained ½ strength Murashige and Skoog

media (MS, or modified MS salts with no nitrogen) supplemented by 10 g l^{-1} sucrose (½MS10), 1.5% Bacto-Agar. Sealed plates were transferred to a controlled environment room and grown at 22 ± 2 °C, on a cycle of 16-h light: 8-h dark. For diversity analysis, seedlings were removed from the plates in a class II biological safety cabinet at 21-day postgermination at which stage they had 3–5 leaves, approximately 2 cm long, and 6–7 cm of root. Each seedling was cut vertically from shoot to root tip with a flame-sterilized scalpel to provide two tissue samples at the same time point for culture-independent and culture-dependent analyses.

DNA extraction and 16S rDNA library preparation

PCR-clone libraries were generated to amplify the majority of the 16S rRNA gene, in preference to short-read next-generation sequencing for this previously uncharacterized microbiome. Near-complete 16S rDNA sequences enable accurate taxonomic identification to species level (Yarza *et al.*, 2014) and minimize the risk of chimeric sequences being generated.

Sterilized plant tissues were ground in liquid nitrogen and homogenized prior to DNA extraction to enable bacteria to be isolated from all tissue layers. Samples were ground using the Spex Freezer/Mill 6870 (SPEX SamplePrep, Stanmore, UK), with a 10-min precooling period at the maximum milling speed in 2-min increments.

Bacterial DNA was extracted using the Fast DNA™ SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions with minor modifications. Fresh freezer-ground material was used in place of freeze-dried, but the recommended weight used. In fresh material, this consisted of: 1× whole seedling, 2 × 1 cm lengths of stem/rhizome, 3 × 1 cm lengths of leaf or 4 × 1 cm lengths of roots placed in individual lysis tubes. The DNeasy Plant Mini kit (QIAGEN Inc., Manchester, UK) was used to extract chloroplast DNA from 600 μl of isolated chloroplast tissue. An aliquot of the PCR products was analysed on a 1% agarose gel run at 100v for 45 min. Those analysed reactions showing single bands of the predicted size were either cut from the gel and purified or treated with ExoSAP-IT PCR Product Clean-Up (USB products Affymetrix) to remove impurities.

The 16S rRNA gene sequences (16S rDNA) were amplified using Phusion® High-Fidelity DNA Polymerase (NEB) according to the manufacturer's instructions. A combination of forward and reverse primers was used to determine primer bias effects (Table 1). The final rinses obtained from tissue sterilization were used as DNA template in a PCR reaction with 16S rRNA primers to determine whether all epiphytic bacteria had been removed from the sample. PCR reactions were performed in an ABI 2720 Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA): an initial denaturation step of 98 °C for 30 s was followed by 35 cycles of denaturing at 98 °C for 5 s, annealing at 60 °C for 10 s, extension at 72 °C for 15 s, followed by a final extension step of 72 °C for 7 min.

PCR products were cloned into the pCR®4Blunt-TOPO® using the Zero Blunt® PCR Cloning Kit for sequencing (Invitrogen, California, USA). Incubation following transformation was extended to 90 min on a VWR Incubating Mini Shaker (VWR, Lutterworth, UK) to account for the orbit size and was on Luria–Bertani medium with kanamycin (50 μg ml^{-1}). Plates were incubated at 37 °C for 30 min prior to plating out the transformed *Escherichia coli* in 50 μl aliquots, and plates were incubated overnight at 37 °C.

Table 1 (A) 16S rRNA primer sequences used in the study. (B) Primer combination abbreviations and *expected sequence length*. 27F and 1492R (pair F) were used by Second Genome for PhyloChip analysis (*)

Primer name	Primer sequence 5′-3′
8F	AGAGTTTGATCCTGGCTCAG
27F (degenerate version of 8F)*	AGRGTTTGATCMTGGCTCAG
63F	CAGGCCTAACACATGCAA
338F	ACTCCTAGGGGAGGCAG
1492R*	GGTTACCTTGTTACGACTT
BSR1541/20R	AAGGAGGTGATCCAGCCGCA
1378R	CGGTGTGTACAAGGCCCGGGA

Primer name	63F	338F	8F
1541/20R	A (1478)	B (1203)	C (1533)
1492R	D (1429)	E (1154)	F* (1484)
1378R	G (1315)	H (1040)	I (1370)

Sequencing and analysis

A total of 24 colonies per plate was tested by colony PCR using M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers. PCR was performed in an ABI 2720 Thermal Cycler, and an extended first step at 94 °C for 4 min was implemented to increase physical disruption of the bacterial colony in the initial stage of the PCR. PCR conditions after the disruption step were 40 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and a final extension step at 72 °C for 10 min. PCR products were sequenced using the same forward primer as in the original PCR using an ABI 3730 Genetic Analyser (Applied Biosystems).

Sequences were converted into a FASTA file from ab1 format and analysed in MEGA 5. Vector sequence was removed. To remove chloroplast contamination from the data set, the sequences were aligned using Clustal W against the Miscanthus chloroplast sequence (Pairwise and Multiple Alignment settings: Gap Opening Penalty 15, Gap Extension Penalty 6.66, DNA weight matrix: IUB, transition weight: 0.5′). Chloroplast sequences were removed from the data set, and the remaining sequences aligned against the NCBI GenBank nucleotide and microbial databases using BLAST.

Rarefaction curve. The 16S rDNA sequences were combined into a single file and aligned using MUSCLE [Gap Penalties: Gap Open -400, Gap Extend 0,Max iterations 8 Clustering Method (Iteration 1,2) UPGMB, Clustering method (other iterations) UPGMB, Min Diag Length (lambda) 24]. The aligned data were then inputted into Mothur (Schloss *et al.* 2009) in

FASTA format. The sequences were filtered to remove the '-' characters from alignments which interfere with distance calculations. The filtered data set was used to calculate a distance matrix consisting of pairwise distances between aligned DNA sequences in a lower triangle phylip matrix format. The distance matrix was then clustered into OTU's based on genetic distance. Clustering produced a species abundance file suitable for a species abundance plot, a rank abundance file for plotting a rank abundance plot and a list file indicating the sequences that cluster together within an OTU. The list file was used to calculate rarefaction curve data with a cut-off at 0.03 (97% similarity), and data from the rank abundance file were plotted using Excel.

Sequence identification. Utilizing the local blast tools (Camacho *et al.*, 2008), the sequencing results for the separate tissues were analysed with the microbial 16S rRNA gene database using blastn and megablast. The results from the different blast queries were read using the BioPython NCBI XML parsing modules (Cock *et al.*, 2009). A python script was created that extracts the top match for each of the sequences from the results of the blast query. The script also extracts the bit scores, percentage identities and E scores for each of the top matches. Sequences that had no matches were also recorded during parsing.

The development of metagenomics technologies has allowed the sequencing of microorganisms previously uncultured and therefore uncharacterized (Rinke *et al.*, 2013). These large numbers of uncultured sequences have been grouped into candidate phyla, including BRC1 and WPS-2. BRC1 is a candidate phylum where genomic data were isolated from samples originating in bulk soil and rice roots. WPS-2 samples have wider origins from soil (Nogales *et al.*, 2001) to ancient dental calculus deposits (Adler *et al.*, 2013).

Endophyte Community Profiling via PhyloChip™

To overcome the limitations of the clone library approach, the PhyloChip™ G3 Assay (Second Genome) was employed to identify the bacterial diversity present in *M. sinensis* and *M. sacchariflorus* seedlings, grown as described in section 1. The G3 PhyloChip™ technology contains 1.1 million 16S rDNA sequence probes representing 60 000 microbial taxa. According to the manufacturer, the technology can be expected to identify a maximum of 3000 taxa from a soil sample, 1800 taxa from a ground water sample and 400 taxa from plant sample [personal communication Alex Probst, Second Genome]. Bacterial DNA was isolated from 21-day-old seedlings (grown under sterile conditions from surface-sterilized seed with sufficient or low nitrogen) and sent to Second Genome for analysis. Bacterial 16S rDNA gene amplicons (primers 27F and 1492R primers, Table 1) were fragmented, biotin-labelled and hybridized to the PhyloChip™ Array, version G3. PhyloChip arrays were washed, stained and scanned using a GeneArray® scanner (Affymetrix). Each scan was captured using standard Affymetrix software (GeneChip® Microarray Analysis Suite). Following QC filtering, Second Genome's PhyCA-StatsTM analysis software package was used for multivariate statistical analysis.

Culturing bacteria from Miscanthus tissues

Surface-sterilized plant materials were placed on agar media plates to culture endophytic bacteria. Leaf, root and seedling materials were lightly ground with mortar and pestle to break the outer tissue layers before being placed onto the media. Stem and rhizome pieces were split horizontally and placed with the internal tissue touching the media. Final rinses from surface sterilization were plated out onto agar media to test for growth of any residual epiphytic bacteria. To isolate the maximum diversity of bacteria from within Miscanthus tissues, a range of nonspecific media was used as follows: nutrient agar (NA; Melford Laboratories, Ipswich, UK), Pseudomonas agar with CFC supplement (PA; Melford Laboratories), mannitol salt agar (MSA; Melford Laboratories), cyanobacteria blue-green medium BG11 (BG, Sigma-Aldrich) with agar and Wilkens–Chalgren anaerobic agar (WC; Melford Laboratories). Plates were incubated at room temperature for at least 72 h and checked daily for bacterial growth. For anaerobic conditions, plates were placed inside culturing jars (VWR) and an anaerobic environment created using GasPak EZ Container System Sachets (Becton, Dickinson and Company, Oxford, UK). The environment was monitored with GasPak anaerobic environment indicator strips (Becton, Dickinson and Company).

Microscopy

General preparation of germinated *M. sacchariflorus* seedlings for microscopy. Dry *M. sacchariflorus* seeds were surface-sterilized in 10% sodium hypochlorite solution for 15 min, rinsed in sterile double distilled water (sdH_2O) and immersed in 70% ethanol for 15 min with regular swirling, then rinsed three times in sdH_2O. They were imbibed in more changes of sdH_2O at ambient temperature until germination was observed. The radicle emerged after 2–5 days.

For LIVE/DEAD BacLight™ Staining. A 2X working solution of the LIVE/DEAD BacLight™ staining reagent mixture was made by dissolving equal quantities of the two components according to the manufacturer's instructions in 5 ml of 0.22 μm sdH_2O. Just-germinated unfixed seedlings were dissected so that the root tips were separated from the seed coat and endosperm on a plain glass microscope slide. Drops of staining solution were added, and the preparation was pressed between the slide and a new coverslip. The resulting squashes were immediately examined and photographed with bright field illumination and ultraviolet light using a Leica LMD6000B microscope. Digital images were acquired.

Ruthenium Red staining of bacterial structures in M. sacchariflorus seedlings. 0.1% Ruthenium Red (Sigma-Aldrich Technical Grade) was added to 2.5% glutaraldehyde in 0.1M sodium cacodylate (both Agar Scientific, Standstead, UK) pH 7.2 to make a deep purple solution. This was used instead of plain fixative. Once germinated and fixed, there were two changes of sodium cacodylate wash buffer at pH7.2 and then a secondary fixative of 1% osmium tetroxide (Agar Scientific) in 0.1M sodium cacodylate at pH 7.2. Two more rinses in wash buffer were followed by an sdH_2O bath and then dehydration in an aqueous alcohol series (30%, 50%, 70%, 95% and 100%) for at least 1 h in each mixture.

Fixed and dehydrated seedlings for sectioning, light microscopy, Raman microscopy and transmission electron microscopy (TEM) were embedded in resin. Infiltration with resin was achieved using mixtures of 2:1, 1:1 and 1:2 of ethanol and LR White (hard grade) resin for at least 3 h each then 100% resin. The samples were transferred to size 0 gelatine capsules, filled with resin and polymerized overnight at 60 °C.

TEM examination of extracted bacterial cultures was conducted broadly as per Evans *et al*., (2012), but the procedure was modified to substitute these steps: the samples were centrifuged and the supernatant discarded. They were then resuspended in 100 μl agarose solution at 25 ^{0}C and placed in a refrigerator to gel at 4 ^{0}C overnight. The next day the gels were cut from the Eppendorf tubes and transferred into 1 ml wash buffer in 5-ml glass vials with push-on lids at 4 °C. After 30 min, they were placed in fresh wash buffer.

Light microscopy. 2-μm-thick sections were cut which contained tissues of interest and dried down on drops of 10% ethanol on glass microscope slides on a hot plate @ 60 °C. They were stained with AMB stain (Azure II & Methylene Blue), covered in a coverslip with Eukitt mountant (O. Kindler GmbH, Germany) and imaged using a Leica DM6000B microscope fitted with a Hitachi digital camera system.

Raman Microscopy. Unstained 2-μm-thick sections of root tips were cut for Raman microscopy and performed as per Webb *et al*. (2013) with the exception that multivariate images collected from the sections were visualized by their Raman emission at 997 cm^{-1} which is indicative of the presence of dipicolinic acid and its calcium salt known to be present in bacterial endospore cores at concentrations of up to 20%.

Transmission Electron Microscopy. Ultrathin 60- to 80-nm sections were cut on a Reichert-Jung Ultracut E Ultramicrotome with a Diatome Ultra 45° diamond knife and collected on Gilder GS2X0.5 3.05-mm-diameter nickel slot grids (Gilder Grids, Grantham, UK) float-coated with Butvar B98 polymer (Agar Scientific) films.

Immunogold labelling was performed according to the method of Webb *et al*. (2013) with the following modifications:

- To determine whether endospore-like structures in Miscanthus bacterial cultures were Bacilli, sections were immunogold labelled using anti-Bacillus primary antibody (AbCam ab20556) 1:100 and the secondary antibody, EM.GAR.10 (BBI Solutions Ltd, Cardiff, UK) goat anti-rabbit IgG conjugate, 10 nm gold 1:200.
- To determine whether bacteria-like structures in bacterial cultures or in planta were Clostridium species, sections were immunogold labelled by the same method using anti-Clostridium primary antibody (AbCam ab20447) with secondary antibody as above.

All TEM sections, whether or not they were immunogold labelled, were double-stained with uranyl acetate (Agar Scientific) and Reynold's lead citrate (TAAB Laboratories Equipment Ltd, Aldermaston, UK) and observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV. The resulting images were photographed using Carestream 4489 electron microscope film (Agar Scientific) developed in Kodak D-19 developer for 4 min at 20 °C, fixed, washed and dried according to the manufacturer's instructions. The resulting negatives were scanned with an Epson Perfection V800 film scanner and converted to positive images.

Results

Bacterial endophytes were present in all mature M. sinensis organs, and in seedlings grown in a sterile environment

A diverse bacterial population was identified by 16S rDNA clone library analysis using 24 clones from each of nine primer combinations (Table 1), from all mature tissues (leaf, stem, rhizome and root) of an *M. sinensis* plant and seedlings grown from surface-sterilized *M. sinensis* seed grown in a sterile environment ('seedlings'). A 16S rDNA clone library was also prepared from isolated chloroplasts using the nine primer pairs, and sequences homologous to these were removed from subsequent analysis. To analyse diversity as opposed to abundance, replicate sequences were removed. Different phyla were identified by each primer pair in the different samples (Table 2), and the sequences from all nine primer combinations were combined for further analysis. In all cases, PCR from the final tissue sterilization washes did not produce an identifiable DNA fragment, and no microbial growth was detected on media plates. It was therefore assumed that the bacteria identified from the tissue samples were endophytic, or very closely associated with the epidermal tissue, and are henceforth referred to as endophytes.

Where there was a match to an existing GenBank bacterial species record, the ID for endophytes from mature species matched GenBank records at 91 to >99%. However, 6–33% of the sequences did not match to an existing species record and are henceforth termed 'unknown' (Table 3). All sequences from root, rhizome and leaf tissue aligned to NCBI GenBank records with a high degree of similarity (E values of ~0). However, 5 sequences from stem and over 40 sequences from the seedling had much higher E values, indicating that they did not accurately match existing records (Fig. 1). Despite a similar frequency of bacterial sequences which could not be allocated to a phylum from both seedling and root, all the root sequences had previous entries on the databases, in contrast to the seedling sequences, many of which did not (Fig. 1). Clear differences in the bacterial diversity at phyla, family and species levels were identified between samples, both in terms of total number and presence/absence of different groups (Table 3).

At phyla level, the seedling tissue hosted the most diverse population of endophytes with 18 of the 20 phyla represented. Of these six bacterial (Caldithrix,

Table 2 Phyla identified in Miscanthus samples using nine different primer combinations (see Table 1 for details). No single primer pair identified the full diversity present in any one sample

Phyla	Leaf	Stem	Root	Rhizome	Seed
Actinobacteria	ABEH		D	CDEFG	BCD
Alphaprotebacteria	ABCDEG	BCDE	DEGH	CDFG	ABCDE
Bacteroidetes	E	BEG	EHGI	E	BEI
Betaproteobacteria	B	B	E	BCDE	BDE
Chlorobi		A			GH
Chloroflexi					H
Crenarchaeota					GH
Cyanobacteria	ABCDEFGHI	ABCDEFGHI	GHI	ABCDEFGHI	ABCDEFGHI
Deferribacteres					H
Deltaproteobacteria		D	H	G	G
Epsilonproteobacteria				BCDF	
Euryarchaeota				GH	GH
Firmicutes	AB	ABG	DEGH	ADH	ABE
Fusobacteria					DF
Flavobacteria		H			
Gammaproteobacteria	ABCDEFGHI	ABCDEFGHI	ABCDEFGHI	ABCDEFGHI	ABCDEFGHI
Planctomycetes	AFGHI	ABEFHI	DGH	BCDEFGH	ABCDEFHI
Spirochaetes					BD
Thermotogae					H
Synergistetes		D	D	D	D
Unclassified	ABCDEFGHI	DEI	ABCDEFGHI	ABCDEFGHI	ABCDEFGHI

Chloroflexi, Deferribacteres, Fusobacteria, Planctomycetes, Spirochaetes) and one archaeal phyla (Crenarcheotea) were uniquely present in seedlings (although only represented by a single, or in one case, two individual sequences). Only Epsilonproteobacteria and Thermotogae were absent from seedlings, and 11 phyla were common to mature tissues and seedlings (Table 3). Gammaproteobacteria, Firmicutes and Alphaproteobacteria were the three phyla most represented in the seedling. Both proteobacteria groups were also common in the mature tissues; Alphaproteobacteria represented the most common phyla in aboveground tissues and Gammaproteobacteria belowground; however, the Firmicutes were found only in the stem and rhizome. A total of three phyla were present only in mature tissues, Deltaproteobacteria and Thermotogae which were present only in low numbers in the leaf and root, respectively, and the Epsilonproteobacteria which were the second most abundant phyla in the rhizome. Whilst the mature tissues were broadly similar in terms of phyla frequency (leaf and root sequences matched to seven different phyla, stem and rhizome sequences matched to nine phyla), the representation of the phyla was different in each tissue. No Actinobacteria and Betaproteobacteria were identified in stem tissue; however, Chlorobi sequences were present in the stem but not in any other mature tissue. Epsilonproteobacteria were identified exclusively in rhizome tissue, one of only three phyla not represented in the seedling. The leaf and root contained the fewest phyla; the archaeal phyla Euryarchaeota was absent from both leaf and root, Firmicutes were absent only from the root, and Synergitetes were not detected in the leaf. At family level, the picture was more complex. A total of 49 families was unique to the mature tissues, 31 were unique to the seedlings and 15 were common to both mature and seedling tissues (Table 3). The 16S rDNA sequences from the seedling represented the largest number of families from a single sample, represented both a larger number of species within each phyla as well as bacterial groups that were not present in the mature plant tissues. The leaf tissue had the lowest number of different bacterial families identified with only 14 bacterial families present, compared to 28 families in the rhizome and 47 families in the seedling (Table 3). The majority of families were represented by only one or two sequences (species); however, a relatively small number of families contained greater diversity. Within the Alphaproteobacteriaceae, there were up to eight unique sequences per family (in the Sphingomonadaceae), up to nine sequences per family in the Betaproteobacteriaceae (Oxalobacteraceae) and 14 different members of the Cyanobacteria. The group with the largest number of presumed species was the pseudomonads with 33 unique sequences, primarily in the root and seedling. Unlike the Cyanobacteria which were ubiquitous within

Table 3 Phyla and families of bacterial endophytes and their location identified using 16S rDNA clone libraries. A total of 216 clones per tissue were subjected to colony PCR, the products sequenced and submitted to BLAST for identification. **Archaeal phyla*, **((unknown/(unknown+known)*100)

Phyla	Family	Leaf	Stem	Rhizome	Root	Seedling	Sum Family	Sum Phyla
Actinobacteria	Acidimicrobidae				1		1	
	Actinosynnemataceae	1					1	
	Cellulomonas				1		1	
	Corynebacteriaceae					2	2	
	Geodermatophilaceae	1			1		2	
	Intrasporangiaceae				1		1	
	Microbacteriaceae			2		1	3	
	Mycobacterium					1	1	
	Mycoplasmataceae			1			1	
	Nakamurellaceae	1			1		2	
	Patulibacteraceae					1	1	
	Propionibacteraceae					2	2	
	Streptomycetaceae				1		1	19
Alphaproteobacteria	Acetobacteraceae		2			4	6	
	Alteromonadaceae				1		1	
	Anaplasmataceae			1	1		2	
	Beijerinckiaceae		1				1	
	Bradyrhizobiaceae	1			2		3	
	Brucellaceae		1			1	2	
	Caulobacteraceae				1		1	
	Holosporaceae	1		1	1		3	
	Methylobacteriaceae	2	1	1		1	5	
	Methylocystaceae					1	1	
	Rhizobiaceae	1			4		5	
	Rhodobacteraceae		1				1	
	Rickettsiaceae			1			1	
	Sneathiellaceae	1	1	1			3	
	Sphingomonadaceae	1	6		1		8	43
Bacteroidetes	Chitinophagaceae					2	2	
	Cyclobacteriaceae				1		1	
	Flavobacteriaceae		1			1	2	
	Prevotellaceae					1	1	
	Sphingobacteriaceae	1			2	2	5	
	Unclassified Bacterioidetes			1			1	12
Betaproteobacteria	Comamonadaceae				1		1	
	Methylophilaceae					1	1	
	Oxalobacteraceae	1		3	5		9	11
Caldithrix	Unclassifed Caldithrix					1	1	1
Chlorobi	Chlorobiaceae		1			1	2	2
Chloroflexi	Anaerolineaceae					1	1	
	Unclassifed Chloroflexi					1	1	2
*Crenarchaeota**	*Thermoproteaceae*					*1*	*1*	*1*
Cyanobacteria	Cyanobacteria	3	3	3	2	3	14	
	Prochlorococcus				1		1	
	Synechococcaceae	1	1	1		1	4	19
Deferribacteres	Deferribacteraceae					1	1	1
Deltaproteobacteria	Bacteriovoracaceae		1				1	
	Desulfuromonadales			1			1	
	Phaselicystidaceae					1	1	3
Epsilonproteobacteria	Campylobacterales			1			1	1

(continued)

Table 3 (continued)

Phyla	Family	Leaf	Stem	Rhizome	Root	Seedling	Sum Family	Sum Phyla
*Euryarchaeota**	Halobacteriaceae					1	1	
	Methanobacteriaceae					1	1	
	Methanococcaceae		1				1	
	Methanosarcinaceae					2	2	
	Microbacteriaceae			1			1	
	Micromonosporaceae			1			1	
	Mycobacteriaceae			1			1	
	Promicromonosporaceae			1			1	
	Unclassified Euryarchaeota			1			1	10
Firmicutes	Bacillaceae	1		4		1	6	
	Clostridiaceae		1	1		4	6	
	Halanaerobiaceae			1			1	
	Halobacteriaceae					1	1	
	Lachnospiraceae					1	1	
	Lactobacillaceae			1			1	
	Peptococcaceae			1			1	
	Staphylococcaceae					1	1	
	Streptococcaceae					1	1	
	Thermoanaerobacteraceae		1				1	
	Veillonellaceae			1		1	2	
	Unclassified Clostridiales					1	1	23
Fusobacteria	Fusobacteriaceae					1	1	1
Gammaproteobacteria	Coxiellaceae					1	1	
	Desulfovibrionales					1	1	
	Enterobacteriaceae			2		3	5	
	Legionellales					1	1	
	Methylococcaceae					3	3	
	Moraxellaceae					1	1	
	Pseudomonadaceae		1	9	13	10	33	
	Xanthomonadaceae					1	1	46
	Unclassified Gammaproteobacteria		1	1			2	
Planctomycetes	Planctomycetaceae					1	1	1
Spirochaetes	Spirochaetaceae					1	1	1
Synergistetes	Synergistaceae		1	1		2	4	4
Thermotogae	Thermotogaceae				1		1	1
	Total Family	14	18	28	21	47	85	
	Total Phyla	6	9	11	7	18		19
	Total unknown (no BLAST hit)	1	3	7	20	36		
	% Unknown**	5.6	10.3	13.5	31.7	33.0		

the plant, notably no pseudomonads were identified in the aboveground leaf and stem tissues.

The root tissue contained a similar number of unique sequences to the rhizome (43 and 45, respectively), representing 21 families and seven phyla in the root, and 28 families and 11 phyla in the rhizome. Leaf tissue contained the least diversity at all levels with only 17 unique sequences, including a single sequence not identified in another tissue, representing 14 families and six phyla. This may be due at least in part to the high frequency of sequences removed as matches to chloroplast. Stem tissues, with 26 unique sequences over 18 families and nine phyla, contained a majority of species in the Alphaproteobacteria. The seedling showed the highest diversity with 73 BLAST hits identified to 47 families and 18 phyla. (Table 3). At least one sequence was identified in each sample which did not match any sequence in GenBank and was hence termed 'unknown'. Leaf, stem and rhizome samples had the lowest number of 'unknown' 16S rDNA sequences with 1, 3 and 7, respectively, whilst root samples contained 20 unknown sequences and seedling had 36.

The 16S rDNA clone library approach used here, whilst enabling high-quality Sanger sequencing of the majority of the 16S rRNA gene for high-quality identification by homology with respect to short-read NGS

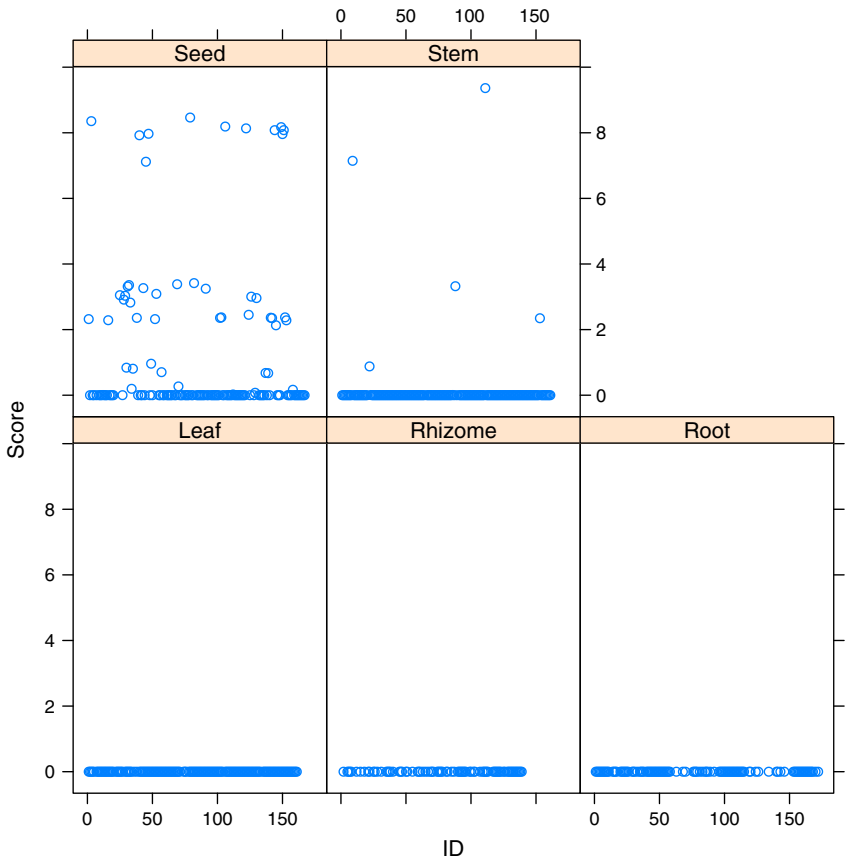

Fig. 1 E values from GenBank matches to 16S rDNA sequences of bacterial endophytes isolated from different Miscanthus plant parts when aligned using the BLAST algorithm, plotted by tissue type. E = 0 indicates a perfect match.

techniques, was a low throughput methodology, based on the sequencing of 216 clones from each tissue type. Production of a rarefraction curve by calculating the OTU's in the data set using MOTHUR indicted that this depth of sampling had not identified the full diversity present in each tissue (Fig. 2).

Bacteria in different seedling populations were similar but not identical and showed some variation in response to nutrient status

A hybridization-based method (PhyloChip™) was employed to further analyse the bacterial populations within Miscanthus seedlings. This method, although based on a single primer pair, enabled identification of all 16S rRNA sequences present on the chip, and so represented deeper sampling depth than the 16S rDNA clone library approach, in which only 216 clones per sample were sequenced. PhyloChip analysis also enabled direct comparison of the bacterial populations present within seedlings, and, at phyla level at least, this indicated that a very similar population was present in individual seedlings, not only within seed from the same panicle but also within seed of different species (*M. sinensis* and *M. sacchariflorus*, Table 4). PhyloChip analysis identified up to 44 distinct bacterial families compared to the 47 identified in the seedling tissue identified by sequencing the 16S rDNA clone library, despite the use of a single primer pair as opposed to the nine different pairs used for the clone libraries. An additional 19 groups were identified but could not be identified to a known family. There were between 27 and 45 groups, including the unclassified ones, per seedling (Table 4). In common with the clone library approach, sequences were identified by PhyloChip which could not be identified beyond phyla or family level. However, anything not present on the chip could not be recognized by this method, so some of the 'unknown' strains identified by the 16S rDNA clone library approach would have been missed. Although there were no large differences overall, of 63 groups identified by PhyloChip in *M. sacchariflorus* seedlings, 10 groups were present only in N+ samples (Streptomycetaceae, Cellulomonadaceae, Pelagibacteraceae, unclassified Sphingobacteriales, Chitinophagaceae, Oxalobacteraceae, Desulfobacteraceae, Syntrophobacteraceae, Spirochaetaceae and

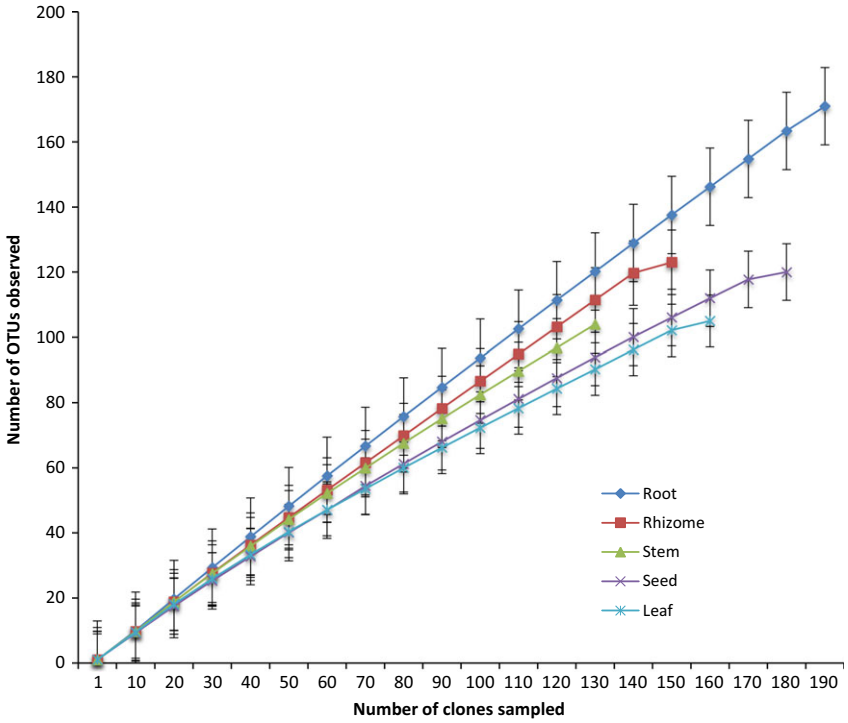

Fig. 2 Rarefaction curve of OTUs observed vs. number of clones, by sample.

Mycoplasmataceae) and six groups were present only in N− samples (Hyphomicrobiaceae, Methylobacteriaceae, Sphingomonadaceae, Bacillaceae, unclassified Firmicutes and unclassified Alteromonadaceae). However, six of these were present in only one of the four seedlings within the treatment (Table 5).

Numerous endophytic bacteria were culturable and readily able to enter dormant states well adapted for seed transmission

Endophytic bacteria were isolated from the same mature *M. sinensis* tissues and seedling tissue as those subjected to 16S rDNA analysis. The 16S rDNA identification of the bacterial strains indicated that, as predicted, the diversity of bacteria isolated in culture from the tissues was lower than that indicated by molecular methodology (Table 6), despite the use of multiple isolation conditions (different media, +/− oxygen, length of time of culture). The isolates obtained derived from five bacterial families, all of which were represented in the clone libraries. However, we were unable to obtain a PCR product from many of the cultures, indicating that these strains would not have been identified by our 16S rDNA clone library approach. Both anaerobic and aerobic species were cultured, but only very few anaerobes (*Clostridria* sp.) were isolated, and these originated exclusively from the stem. In the 16S rDNA clone libraries, sequences from anaerobic bacterial groups were identified in the stem and seedling (*Clostridria*, Chlorobi, Synergistetes) and Thermotogae were identified in the root tissue sample Table 3.

Some of the isolates were readily able to form endospores and other dense refractive structures following a period of nutrient depletion (Fig. 3), thereby providing a mechanism by which the bacteria may remain viable within the desiccated seed until it germinates. Although some of the structures were typical of Bacilli known to be spore-forming (confirmed using anti-Bacillus anti-Bacillus primary antibody), a number of the strains we were unable to identify by 16S rDNA analysis also formed dense refractive structures in culture. Some of these displayed immunogold labelling with anti-Clostridium primary antibody (Fig. 3).

Endospore structures were visible in newly germinating seeds; bacterial cells could be observed at high frequency in the root tip and adjacent tissues

Following identification of bacteria in seedlings grown in sterile conditions, microscopy was employed to localize the bacteria within *M. sacchariflorus* seed. At the first sign of germination, as the radicle ruptured the testa (2–5 days following imbibition), seeds were subjected to a range of techniques to visualize and characterize the bacteria present.

Table 4 Phyla and families of bacterial endophytes identified using PhyloChip. 8 *M. sacchariflorus* (Sac) and *M. sinensis* (Sin) seedlings were grown to 3 weeks with sufficient (N+) and low (N−) nitrogen. Seeds of 8 *M. sachhariflourus* and 4 *M. sinensis* seedlings grown with (N+) or without (N−) nitrogen identified using PhyloChip analysis

Phyla	Family	Sac N+ #1	Sac N+ #2	Sac N+ #3	Sac N+ #4	Sac N- #1	Sac N- #2	Sac N- #3	Sac N- #4	Sin N+ #1	Sin N+ #2	Sin N+ #3	Sin N- #1	Sum Families
Acidobacteria	Acidobacteriaceae	1	1	1	1	1	1	1	1	1	1	1	1	2
	Solibacteraceae	1	1	1	1	0	1	1	1	1	1	1	1	
	Unclassified	0	0	0	0	0	0	0	0	0	1	1	0	
	Chloracidobacteria unclassified	1	1	1	1	1	1	1	1	0	1	0	1	
Actinobacteria	Cellulomonadaceae	0	0	1	0	0	0	0	0	0	1	0	1	5
	Microbacteriaceae	1	1	1	1	1	1	1	1	1	1	1	1	
	Micrococcaceae	1	0	0	1	1	1	1	1	0	1	0	1	
	Propionibacteriaceae	0	0	0	0	0	0	0	0	0	0	1	0	
	Streptomycetaceae	0	1	1	1	0	0	0	0	1	1	0	1	
	Unclassified *Actinomycetales*	1	0	1	1	0	1	1	1	0	0	1	1	
Alphaproteobacteria	Beijerinckiaceae	0	0	0	1	1	1	0	1	0	0	0	0	8
	Bradyrhizobiaceae	1	1	0	1	1	1	1	1	0	0	0	0	
	Hyphomicrobiaceae	0	0	0	0	1	1	1	1	0	1	0	0	
	Methylobacteriaceae	0	0	0	0	1	1	1	1	0	1	0	0	
	Pelagibacteraceae	0	0	1	0	0	0	0	0	0	0	0	0	
	Rhizobiaceae	0	0	1	0	0	1	1	1	1	0	0	1	
	Rhodospirillaceae	1	1	1	1	1	1	1	1	1	1	0	1	
	Sphingomonadaceae	0	0	0	0	1	1	1	1	0	0	0	0	
	Unclassified *Alphaproteobacteria*	0	1	1	1	1	1	1	1	0	0	0	1	
	Unclassified *Rhizobiales*	1	1	1	1	1	1	1	1	1	1	1	1	
Bacteroidetes	Chitinophagaceae	1	0	0	1	0	0	0	0	1	0	0	0	4
	Flexibacteraceae	1	1	1	1	1	1	1	1	1	0	1	1	
	Porphyromonadaceae	1	1	1	0	0	0	1	0	0	1	0	0	
	Rikenellaceae	1	1	1	1	1	1	1	1	1	1	1	1	
	Unclassified *Sphingobacteriales*	1	1	0	1	0	0	0	0	1	0	0	1	
Betaproteobacteria	Alcaligenaceae	0	0	0	0	0	0	0	0	0	0	0	0	4
	Burkholderiaceae	0	0	0	1	0	1	0	0	0	0	1	0	
	Comamonadaceae	0	0	0	1	1	1	1	1	0	1	0	0	
	Oxalobacteraceae	1	0	0	0	0	0	0	0	0	0	0	1	

(continued)

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Table 4 (continued)

Phyla	Family	Sac N+ #1	Sac N+ #2	Sac N+ #3	Sac N+ #4	Sac N- #1	Sac N- #2	Sac N- #3	Sac N- #4	Sin N+ #1	Sin N+ #2	Sin N+ #3	Sin N- #1	Sum Families
Chloroflexi	Anaerolinaceae	1	1	1	1	0	1	1	1	1	1	1	1	1
Cyanobacteria	Phormidiaceae	1	1	0	1	0	0	1	1	1	1	1	1	2
	Synechococcaceae	0	0	1	1	0	1	1	0	0	0	1	1	
	Unclassified *Cyanobacteria_1*	1	1	1	1	0	1	1	1	1	1	0	1	
	Unclassified *Cyanobacteria_2*	0	1	1	1	0	1	0	0	1	1	1	1	
Deltaproteobacteria	Desulfobacteraceae	1	0	1	1	0	0	0	0	0	0	0	0	4
	Desulfobulbaceae	0	0	0	0	0	0	0	0	0	0	1	1	
	Nitrospinaceae	0	0	0	0	0	0	0	0	0	0	0	0	
	Syntrophobacteraceae	1	1	0	1	0	0	0	0	1	0	1	1	
	Unclassified *Deltaproteobacteria*	1	1	1	0	0	1	1	1	0	1	1	1	
	Unclassified *Syntrophobacterales*	1	1	1	1	0	0	0	1	1	1	1	1	
Firmicutes	Bacillaceae	0	0	0	0	0	1	0	0	0	0	0	0	2
	Lachnospiraceae	1	1	1	1	1	1	1	1	1	1	1	1	
	Unclassified *Bacillales*	0	0	1	0	0	1	0	1	0	0	0	0	
	Unclassified *Clostridiales*	1	1	1	1	0	1	1	1	1	1	1	1	
	Unclassified *Firmicutes*	0	0	0	0	0	0	1	0	0	0	0	0	
Gammaproteobacteria	Alteromonadaceae	1	1	1	1	0	0	1	1	0	0	1	1	5
	Chromatiaceae	1	1	0	1	1	1	0	1	0	0	1	1	
	Enterobacteriaceae	1	1	1	1	0	1	1	1	1	1	1	1	
	Halomonadaceae	1	1	1	1	0	1	1	1	1	1	1	1	
	Pseudomonadaceae	1	1	1	1	1	1	1	1	1	1	1	1	
	Unclassified *Alteromonadaceae*	0	0	0	0	1	0	0	0	0	0	0	0	
	Unclassified *Gammaproteobacteria*	1	1	1	1	1	1	1	1	1	0	1	1	
	Unclassified *Oceanospirillales*	1	1	1	1	1	0	0	0	1	1	1	1	
Gemmatimonadetes	Gemmatimonadaceae	0	0	0	1	1	1	1	1	0	0	0	0	1
Planctomycetes	Planctomycetaceae	1	1	1	1	1	1	1	1	1	1	1	1	1
Proteobacteria	Unclassified *Proteobacteria*	1	1	1	1	1	1	1	1	1	1	1	1	
Spirochaetes	Spirochaetaceae	1	0	1	1	0	0	0	0	1	1	0	1	1
Tenericutesæ	Acholeplasmataceae	1	1	1	1	0	1	1	0	1	0	1	0	3
	Erysipelotrichaceae	1	1	1	1	1	1	1	1	0	1	1	1	
	Mycoplasmataceae	1	0	0	1	0	0	0	0	1	1	1	1	
Verrucomicrobia	Verrucomicrobiaceae	1	1	1	1	1	1	0	1	1	1	1	1	1
WPS-2	Unclassified *WPS-2*	0	0	0	1	0	1	1	1	0	1	0	0	
BRC1	Unclassified *BRC1*	1	1	1	1	1	1	1	1	0	1	1	1	
	Total Phyla	16	14	16	18	13	17	16	17	14	17	15	16	18
	Total Families	39	35	38	45	27	41	38	39	30	35	34	41	44
	Total Unclassified	12	13	13	14	7	12	12	12	10	13	12	15	19

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Table 5 Seed endophyte families affected by seedling N status, as determined by Bray–Curtis dissimilarity analysis. Of 63 groups identified by PhyloChip in Miscanthus sacchariflorus seedlings, 10 were present only in N+ samples and six present only in N− samples. Three of each (indicated in italics) were present in only one of the four seedlings within the treatment

Present only in N+ seedlings		Present only in N− seedlings	
Actinobacteria	Streptomycetaceae	Alphaproteobacteria	Hyphomicrobiaceae
Actinobacteria	*Cellulomonadaceae*	Alphaproteobacteria	Methylobacteriaceae
Alphaproteobacteria	*Pelagibacteraceae*	Alphaproteobacteria	Sphingomonadaceae
Bacteroidetes	Unclassified Sphingobacteriales	*Firmicutes*	*Bacillaceae*
Betaproteobacteria	Chitinophagaceae	Firmicutes	*Unclassified Firmicutes*
Betaproteobacteria	*Oxalobacteraceae*	*Gammaproteobacteria*	*Unclassified Alteromonadaceae*
Deltaproteobacteria	Desulfobacteraceae		
Deltaproteobacteria	Syntrophobacteraceae		
Spirochaetes	Spirochaetaceae		
Tenericutesæ	Mycoplasmataceae		

Light microscopy revealed the presence of numerous refractive bodies, similar to those seen in cultures, both within the embryo itself and outside in the cavity between the embryo and the seed coat, which were hypothesized to be bacterial endospores (Fig. 4). Dipicolinic acid is a major component of the endospore coat which is released from its bound form upon germination of the spore. Using Raman microscopy, a dipicolinic acid signal was localized to the root tip, especially the root cap, and the cavity between the embryo and the seed coat at the earliest stage of germination (Fig. 5). At the same stage, application of an alive/dead stain revealed an abundance of microbial activity in the space adjacent to the emerging radicle (Fig. 6), indicating different locations of two different bacterial populations: endospores within the embryo and living cells in the space outside the embryo. Structures within root tip tissues displaying similarity to the refractive structures seen in the cultured endophytes were confirmed as bacterial by immunogold labelling both in culture and *in planta* (Figs 3 and 4).

Discussion

Miscanthus hosts diverse populations of bacteria in all mature organs and in seedlings grown from surface-sterilized seed

Bacterial populations were present in all samples tested (root, rhizome, stem, leaf and seedling); however, the diversity in *M. sinensis* seedlings was not equivalent to those in the mature *M. sinensis* plant tissues (Table 2). A total of 49 families identified by 16S rDNA analysis were unique to the mature tissues, 31 were unique to the seedlings, and 15 were common to both mature and seedling tissues. Whilst it was possible to sample whole seedlings as they were relatively small, mature Miscanthus is a very large plant and so the samples of mature organs may under-represent the diversity present, in particular certain tissues that were recalcitrant to grinding. Isolates were cultured from the same samples, enabling a comparison of the techniques and further characterization of the cultured species. Although culturing identified fewer bacteria than were identified by the culture-independent methodology, it also revealed that not all cultured bacteria could be identified using the nine 16S rRNA gene primer pairs used in this study. Of those which could be identified, members of all five families cultured (Oxalobacteraceae, Bacillaceae, Clostridiaceae, Enterobacteriaceae and Pseudomonadaceae) were also identified by the two 16S rDNA-based techniques. Comparison (in seedlings only) of the bacterial populations identified by the two culture-independent methodologies indicated that neither had captured the full diversity present: six phyla (Caldithrix, Chlorobi, Deferribacteres, Fusobacteria, Spirochaetes and Synergistetes) were identified uniquely by the 16S rRNA gene library, four phyla (Acidobacteria, Gemmatimonadetes, Tenericutes, Verrucomicrobia, plus WPS-2 and BRC1) were unique to the PhyloChip and 10 phyla (Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Chloroflexi, Cyanobacteria, Deltaproteobacteria, Firmicutes, Gammaproteobacteria and Planctomycetes) were common to both, including the three phyla represented by the cultures. Of the two archaea groups (Crenarchaeota and Euyarcharaeota) identified by 16S rDNA clone sequencing, only the latter was identified by PhyloChip. These differences may be due to insufficient sampling depth in the clone library approach, as indicated by the rarefaction curve (Fig. 2), and primer specificity resulting in whole groups of bacteria not being identified, especially in the PhyloChip analysis which was based on a single primer pair. Nine primer pairs, including the one used for the PhyloChip analysis, were used in the 16S rDNA study to maximize the number of bacterial groups identified, and this

Table 6 Bacterial endophytes of Miscanthus identified by culturing and 16S rDNA amplification and sequencing. Anaerobes are marked with an asterisk*

ID (16S rDNA)	Phylum	Family	Leaf	Stem	Rhizome	Root	Seedling	Sum ID	Sum Family	Sum Phyla
Massilia sp.	β proteobacteria	Oxalobactereaceae					1	1	1	1
Bacillus barbaricus	Firmicutes	Bacillaceae		1				1	19	23
Bacillus cereus							1	1		
Bacillus gelatini				1				1		
Bacillus megatarium				2				2		
Bacillus sp.				3		3		6		
Bacillus subtilis				3				3		
Bacillus thuringiensis			1	2				3		
Bacillus weihenstephanensis					1			1		
Lysinibacillus fusiformis						1		1		
*Clostridium algidixylanolyticum**		Clostridiaceae		3				3	4	
Clostridium sp.*				1				1		
Ewingella americana	γ proteobacteria	Enterobacteriaceae					1	1	8	22
Pantoea ananatis						1		1		
Rahnella aquatilis						3		3		
Serratia sp.						3		3		
Pseudomonas		Pseudomonadaceae				1		1	14	
Pseudomonas brenneri							3	3		
Pseudomonas fluorescens						2	1	3		
Pseudomonas rhizosphaerae							1	1		
Pseudomonas sp.						1	3	4		
Pseudomonas veronii					2			2		
Sum ID			1	16	3	15	11			
Total Families			1	2	2	3	4			
Total Phyla			1	1	2	2	3			
Uncultured bacterium							1	1		
Mixed							4	4		
NSS				1		1		2		
No PCR product			1	8	11	19	32	71		

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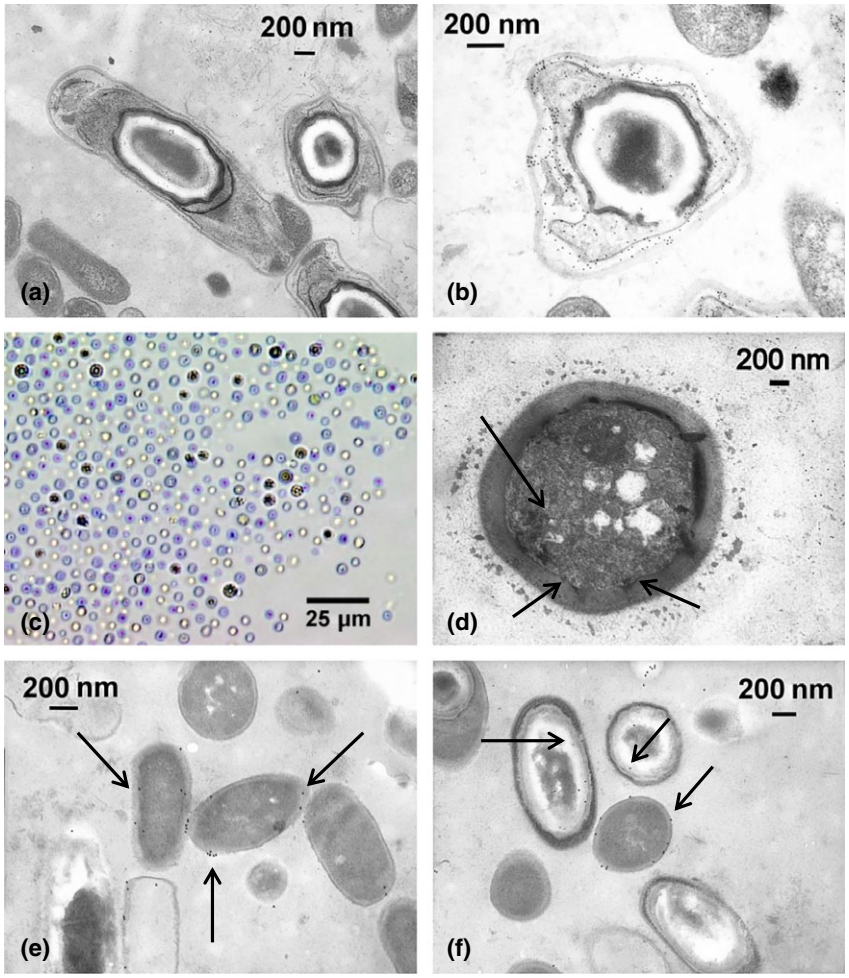

Fig. 3 Cultured endophytic bacteria form various resistant structures in response to nutrient depletion. (a) Spore formation visible in two cells sitting in different planes. (b) Anti-Bacillus cereus immunogold labelling confirmed identity as Bacillaceae. (c) Dense refractive structures were formed by unidentified strains, consistent with structures observed in planta (Fig. 4). (d-f) Three different bacterial strains immunogold labelled using anti-Clostridium primary antibody (highlighted with arrows). (c) and (d) show the same bacterial culture.

revealed the extent of primer bias in commonly used 16S rRNA primer pairs (Table 2). Furthermore, not all tissues would have been equally sampled or exposed to culture medium using these methods. It is therefore likely that a greater diversity of bacteria is present in Miscanthus than is presented here, and that these bacteria form a population which is dynamic, both over the lifetime of the plant, and in response to environmental conditions such as seasonality as has been reported in other species (Islam *et al.*, 2010).

There is much interest in developing bacteria as biofertilizers and biocontrol applications (Farrar *et al.*, 2014). Many Miscanthus endophytes were readily culturable and will be tested for plant growth promotion and antimicrobial activity. Although a broad isolation technique was employed in this study, a proportion of the bacteria present in Miscanthus could require very specific culturing conditions that may not be currently known. Recent developments of single cell culture using gel microdroplets which still allows cell–cell interactions, including through contact with diffusible elements (Dichosa *et al.*, 2014), are particularly likely to be suited to the culture and sequencing of endophytes where a complex population of cells, including from the host plant, can still receive signals and growth factors necessary for growth. Future screens will be targeted to isolate endophytes with specific properties such as biological nitrogen fixation, plant growth promotion, biocontrol and phytoremediation. These properties are of special interest for application to perennial energy crops which are frequently grown under low input conditions on low-quality land, and therefore tolerate various

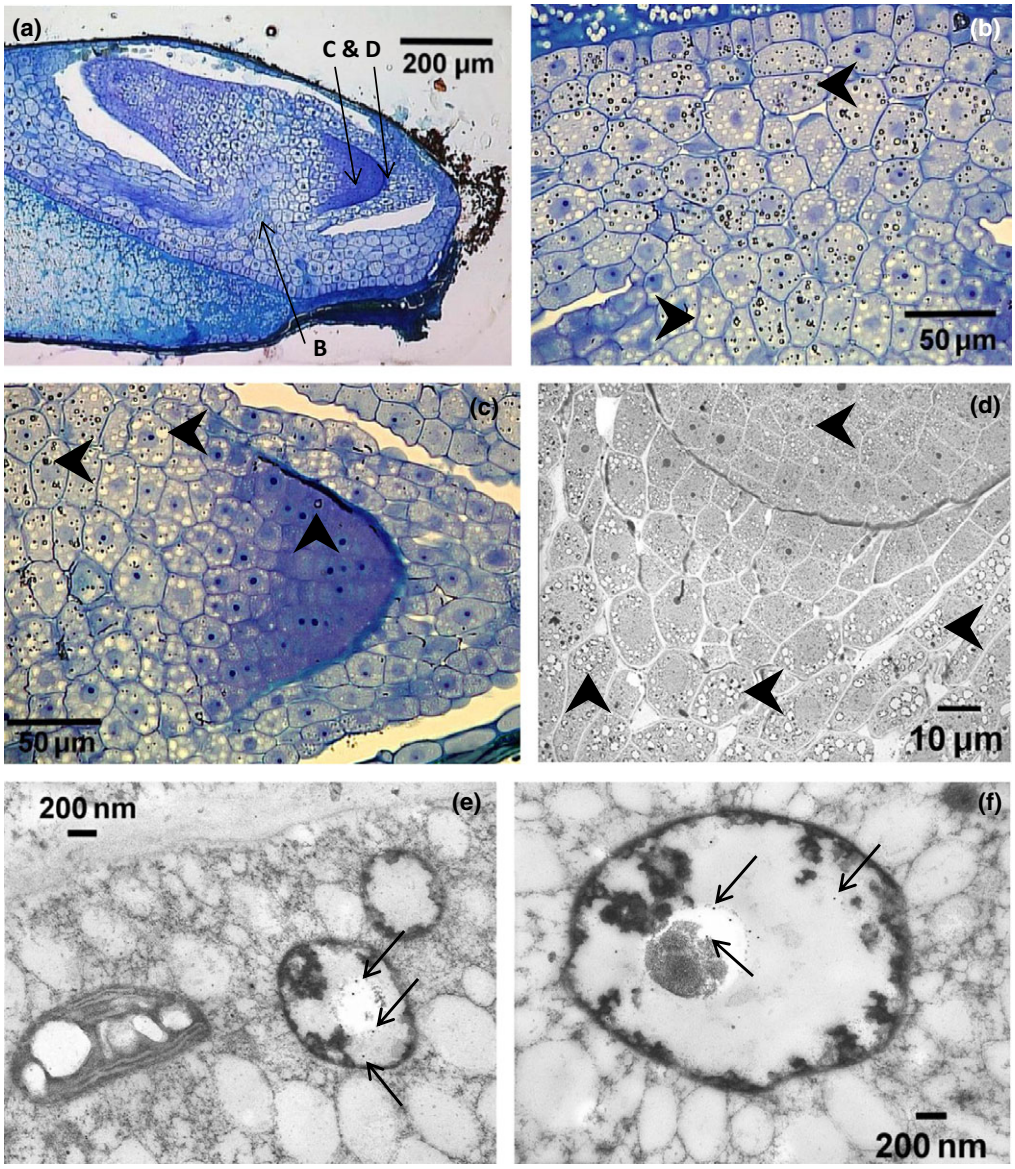

Fig. 4 Refractive bodies and structures hypothesized to be bacterial in origin (highlighted by arrow heads) were observed at high frequency in the imbibed M. sacchariflorus seedling, most notably within the embryo and surrounding coleoptile. (a-c) Ruthenium red staining demonstrating polysaccharide production. (d-f) Electron micrographs. (a) Entire embryo surrounded by coleoptile, with root meristem to the right and endosperm lower left. Arrows indicate the location of subsequent images. (b) and (c) Refractive bodies present within Miscanthus embryo cells at the junction between the embryo and the coleoptile (b) and in embryonic root cells (c). (d) Bacterial structures in the root meristem, root cap and coleoptile. (e) and (f) Bacterial structures at higher magnification immunogold labelled using anti-Clostridium primary antibody (highlighted with arrows).

abiotic stresses over the lifetime of the crop. Previous efforts to isolate endophytes from Miscanthus have largely focused on diazotrophs and have included *Azospirillum*- and *Herbaspirillum*-like species (Kirchhof *et al.*, 1997; Eckert *et al.*, 2001), neither of which were detected in this study, and also Clostridia (Miyamoto *et al.*, 2004), members of which were identified here. In other C4 grasses where a nondirected approach to isolation was employed, more similar strains to the ones identified here were cultured, including *Pseudomonas fluorescens*, *Bacillus subtilis*, *Pantoea*, *Serratia* sp (Gagne-Bourgue *et al.*, 2013).

Vertical transmission of endophytes via the seed

Bacteria have been isolated from the seed of a diverse range of plant species (reviewed by Truyens *et al.*, 2015). However, the majority of these studies were

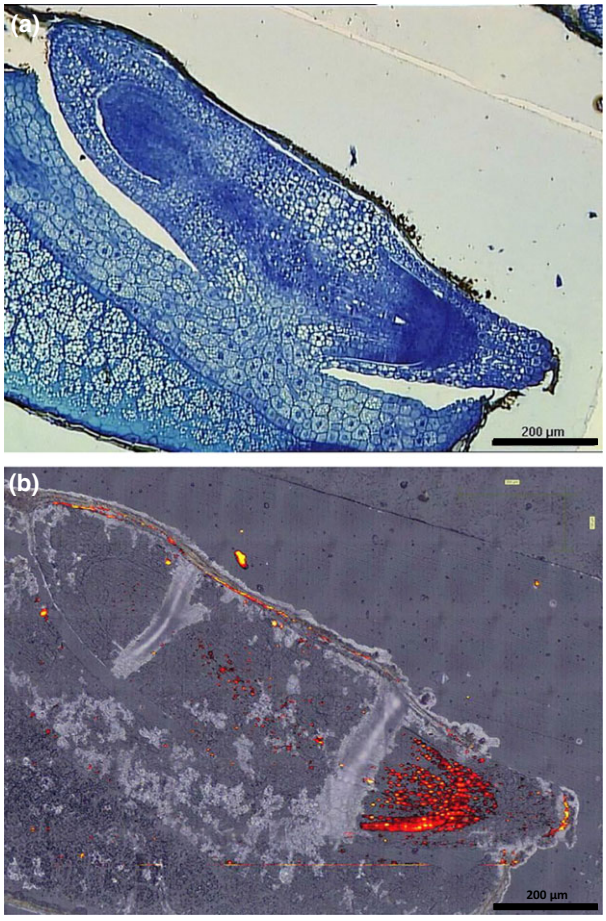

Fig. 5 (a) light microscopy of the imbibed seedling. (b) Raman microscopy of the same seedling displaying signal consistent with dipicolinic acid, a major component of the endospore coat.

limited to culture-based techniques, and some 16S rDNA fingerprinting methodologies. All three methodologies employed in this study confirmed that the seed is host to a diverse population of bacteria, and the use of the 16S rRNA gene libraries and PhyloChip enabled detection of a far greater diversity of phyla than the major phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (subclasses α,β,γ,δ, ε) described previously. The additional phyla were generally represented by fewer sequences than these major groups, again indicating the complexity of the populations present and the need for sampling depth for meaningful assessments of bacterial diversity. PhyloChip analysis indicated that a very similar population was present in each individual seedling (Table 3), consistent with the idea that Miscanthus may exert a degree of control over the bacterial population present in the seed. As the seed was derived from plants grown in pots in the same compost, it is not possible to discount the possibility that this result simply reflects a subset of the bacterial population from the soil in which the parent plant was grown. Indeed, using fluorescently labelled *Burkholderia* phyofirmans strain PsJN, Compant *et al*. (2008) traced bacteria from the soil to the inflorescence in grapevine. Importantly, the bacterial diversity within the seedling provides a route whereby the seed population may move with the developing apical meristem during plant growth and development, even if only in low numbers, to colonize the developing seed upon transition from vegetative to reproductive growth and thereby get transmitted to the next generation. The observation of a dipicolinic acid signal within the central axis of the Miscanthus embryo (Fig. 5) supports this.

It is striking that many of the strains identified as endophytes within Miscanthus are readily capable of forming endospores, spores or other dense structures (potentially cysts) under limited nutrient environments, thereby providing a mechanism by which they might remain dormant and survive within the desiccated Miscanthus seed until germination (Figs 3 and 4). Initial attempts to isolate bacterial sequences from dry seed were unsuccessful, and this could partially be explained by the formation of resistant survival structures such as endospores. Although it cannot be discounted that some or all of these bacteria may be pathogenic, the healthy germination of the Miscanthus seedlings harbouring these bacterial populations, and lack of known bacterial disease in mature Miscanthus plants substantially discounts this. No outgrowth of bacteria was observed when plants were grown from sterilized seed on media, yet cultures were readily obtained from plated mature tissues and ground seedlings. It is tempting to speculate that this may be due to plant regulation of bacterial cell numbers as has been reported previously (Rosenblueth & Martínez-Romero (2006).

The confidence with which sequences match to the entries in the NCBI database is, in effect, an indication of the frequency at which they have been previously identified in other studies. The 16S rDNA sequences generated in this study were analysed using BLAST to both NCBI and GenBank databases to seek species identification. The identification of large number of previously unidentified 16S rDNA sequences from both cultured strains and 16S rDNA sequences, including a number we were unable to identify even to a phyla (Fig. 1, Tables 2 and 4), is indicative of the presence of a large number of endophytic bacteria within Miscanthus seed which may not be found in other environments. The presence of novel bacteria almost exclusively in the seedling indicates the more intriguing possibility of a bacterial population genomically adapted to the symbiotic lifestyle and vertical transmission from one generation of Miscanthus to the next via the seed. All but five of the mature plant

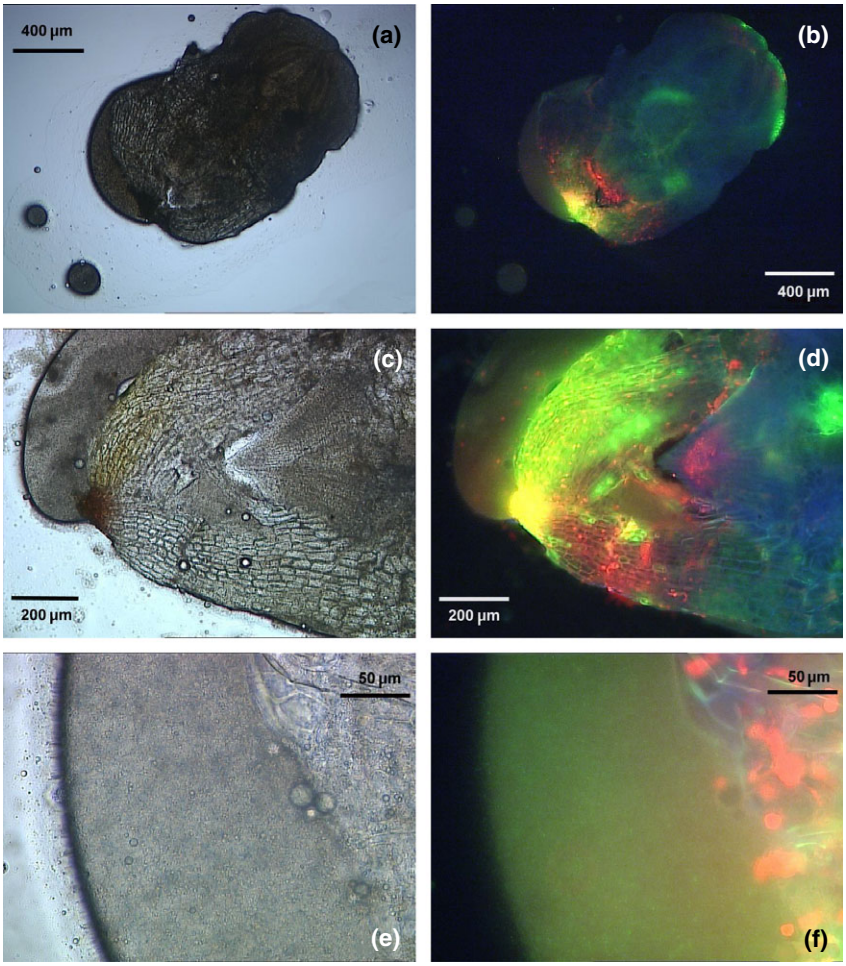

Fig. 6 Bacterial alive/dead stain for bacteria on imbibed Miscanthus seedling. (a) seedling on light microscope after staining (b) seedling under fluorescent light. Green areas show live bacteria and red shows dead bacterial cells. (c) seedling after staining on light microscope showing radicle and embryo with liquid forced from embryo. (d) Seedling under fluorescent showing a large area of live bacterial cells. (e) Liquid from seedling (f) under fluorescence.

sequences showed perfect matches to existing entries; however, the seedling sequences included a large number with no matches, indicating that this population was distinct from the others, potentially including numerous hitherto unidentified species (Fig. 1). Genomic adaptation of bacterial endophytes for a symbiotic life cycle *in planta* may include strategies for vertical transmission via the seed at the expense of competitiveness and ability to survive in most environments outside the plant. The rich diversity in the seed, presumably inherited from the parent plant, indicates an alternative role for seed transmission of bacterial endophytes, not simply as passengers avoiding plant defences, but with an active role, potentially acting primarily during germination and seedling establishment. These bacterial species that have not previously been isolated or sequenced potentially represent a rich untapped resource of novel microorganisms of interest for further study. Genomic analysis of these novel bacteria will provide insights into the genomic adaptation for successful endosymbiosis and may yield routes to generate optimized synthetic bacteria for sustainable crop production.

Seed-transmitted bacteria – a novel role in Miscanthus germination and establishment?

Miscanthus seed is numerous but small, with very little in the way of starch or oil reserves in the endosperm either to support the newly emerging seedling from the transition of heterotroph to photosynthesizing autotroph, or to attract or support a substantial bacterial population. Endophytic bacteria are carried in the seed and visible in seedlings, primarily in the tissues adjacent to the root tip, but also in the cavity between the

embryo and the testa (Figs 5 and 6). The discovery of such a diverse bacterial population within the seed is all the more remarkable given the lack of an obvious carbon source to support them. The use of Raman microscopy to visualize endospores via their dipicolinic acid signal indicated that a localized population of dormant endophytes is present within the embryonic root tip (Fig. 5), and especially the root cap. The alive/dead stain showed an almost inverse signal (Fig. 6), potentially indicating two independent bacterial populations: dormant endospores within the embryo proper and active bacteria localized within the seed tissues proximal to the embryonic root tip. This bacterial activity within a very short period following imbibition is consistent with a hitherto unreported role for these bacteria during germination.

We propose a model whereby Miscanthus actively selects a population of endophytic bacteria to the developing seed, as has been reported in rice (Okunishi *et al*., 2005), grapevine (Compant *et al*., 2008, 2011) and maize (Liu *et al*., 2013), and which are induced to become dormant as the seed matures, consistent with the findings of Okunishi *et al.* (2005). These dormant bacteria germinate along with the seed upon imbibition. Active bacteria were concentrated at the root tip within hours of imbibition (Fig. 6), thereby ensuring that the emerging radicle is bathed in a rich inoculum of seed-derived bacteria as the testa is ruptured during germination, and the mucilage produced by the root tip provides a rich medium in which the bacteria multiply. The bacteria-rich mucilage forms a physical, chemical and microbial sheath around the growing root, modifying the rhizosphere substantially with respect to bulk soil. This physical and biological barrier between the plant and the soil microbiota protects the developing root from soil-borne pathogens, thereby increasing the chances of successful establishment for the seedling. In return, the bacteria reside within the nutrient-rich environment of the plant, and as proposed by Cankar *et al.* (2005) are able to move into new environments via seed transmission. Partial evidence for this hypothesis is emerging: GFP-tagged *Enterobacter asburiae* injected into the stem of maize subsequently colonized the rhizosphere from the root (Johnston-Monje & Raizada, 2011), and seed endophytes were observed in the rhizosphere of plants grown in irradiated soil (Hardoim *et al*., 2012). More work is required to determine whether these bacteria are able to promote seedling growth under conditions of nutrient limitation or other abiotic stresses. However, this role might help to explain the frequent reports of growth promotion in pot trials of young plants not being translated into final yield increases in the field.

Potential evolutionary significance of seed-transmitted endophytes and implications for crop improvement

Miscanthus is an undomesticated perennial grass. It is a colonizing species capable of growth on poor soils and survival over many seasons, including extended periods of abiotic stress. It can be distributed clonally via rhizome, or can establish from its numerous small seeds. Whilst it is tempting for the crop biologist to focus on yield optimization, this is not an evolutionary drive for the plant. Establishment from seed and transmission of genes to the next generation however is essential for survival and hence of major evolutionary significance. It is therefore our assertion that the diverse bacterial population present within the Miscanthus seed is not merely escaping detection by the host defences, but fulfilling a role at the most critical point in the life cycle between dormant seed and the establishment of the Miscanthus seedling. Analysis of bacterial populations is confounded not only by the technical challenges we have discussed, but also by the dynamic diversity of bacteria whereby functionality cannot be determined by taxonomy. It is possible that similar roles may be fulfilled for the host plant via different bacteria under different situations, such as contrasting environments. An initial attempt has been made to determine whether bacterial diversity is common across 'boundaries of evolution, ethnography and ecology' by Johnston-Monje & Raizada (2011), in wild, domesticated and modern maize seed using TRFLP. No significant difference was reported in wild vs. domesticated maize species, and endophytes were found to persist from wild ancestors in domesticated maize. This is consistent with our findings of similar populations of endophytes in the seedlings of *M. sinensis* and *M. sacchariflorus* (Table 4). Further studies using more sensitive methodologies will be required to ascertain the extent to which seed endophytes and/or functions are conserved between all plants, especially other grasses and cereals, and to what extent they have been impacted by domestication and breeding for growth in different locations and for different end uses.

Studies comparing bulk soil, rhizosphere and endosphere tend to assume that the rhizosphere comprises a subset of the bulk soil microbiome, attracted by plant root exudates, from which the plant selectively, at least to some extent, allows ingress of endophytes (horizontal transmission). There is evidence to support aspects of this claim provided by studies following labelled bacteria which can be traced from exogenous inoculation to within plant tissues (James *et al*., 2002; Compant *et al*., 2008). However, this route does not exclude the theory proposed here, that this is only part of the story, and that rather than the rhizosphere being comprised

entirely of (recent) soil microbes, it may largely comprise seed-derived species, potentially transmitted through multiple plant generations (vertical transmission), which have adapted at a genomic level to the endosymbiotic lifestyle. Nonpathogenic bacteria are generally believed to be separated from the host cytoplasm by a plant membrane. We have presented evidence of what we believe to be novel observations of intracellular endophytes in direct contact with the cytoplasm of embryo cells in the newly germinating seedling. We do not yet have evidence about the origin or the fate of these bacteria. Importantly, we are not describing the invagination of the host membrane that occurs with ingress of bacteria from outside the plant, or invasion by a pathogen, but a native seed-transmitted population that is potentially adapted to live its entire life cycle within the plant host. Hence, this represents the first instance of an alternative scenario, consistent with the continuum hypothesis which predicts that vertical transmission of parasites selects for lower virulence (Ewald, 1987). In a related system, Stewart *et al.* (2005) demonstrated that barley stripe mosaic virus virulence was reduced following three generations of vertical transmission in barley. Whole microbes have been incorporated into plant cells as organelles previously and the endophytic relationship may represent a step along this path, with the potential for highly specialized symbiosis or transfer of microbial gene functions into the plant genome over time.

Summary

In this study, we have demonstrated that the bacterial populations within the Miscanthus seed are more diverse than those of the mature plant tissues, indicating that this is a major route for vertical transmission of endophytes from one plant generation to the next, and introducing a novel evolutionary perspective to the plant–bacteria symbiosis via a role in seed germination. Bacterial endophytes that are vertically transmitted may live their whole life cycle within the plant and could specialize to optimize the *in planta* lifestyle, potentially losing the genomic apparatus required for free-living within the soil environment. The identification of numerous novel bacteria from Miscanthus seed provides the opportunity to analyse these genomes for such symbiotic adaptation (Straub *et al.*, 2013b). Whilst the majority of functions essential for plant survival must surely be carried by the plant genome, this provides limitations as it is complex and slow to evolve. A rich microbiome comprising diverse species provides a highly dynamic pool of genomic complexity, capable of rapid evolution and enabling responsive selection of additional functionality depending on the environmental circumstances in which the seedling emerges. Selected or genetically modified bacteria may be used to deliver traits of interest to a host plant.

In order that the plant not be overwhelmed by bacteria, the relationship must presumably be regulated by both host plant and bacterial genomes. Identification and exploitation of the regions of the plant genome regulating these symbiotic relationships will be a major target for 21st century plant science and will provide novel traits and genes for selection in plant breeding.

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